

Taurus Energy AB

Attachment 4

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BUSINESS INFORMATION**

Absence of Antibiotic Resistance Genes in MICROBIAL STRAIN
Taurus04.A.54

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Absence of Antibiotic Resistance Genes in MICROBIAL STRAIN Taurus.A.54

Summary

During the course of construction of PARENT OF MICROBIAL STRAIN Taurus04.A.54 and MICROBIAL STRAIN Taurus04.A.54, antibiotic resistance genes had been used as genetic markers for introduction of DNA. Although the methods used during these construction events eventually result in the complete elimination of these markers, the confirmation of their removal was carried out. Polymerase Chain Reaction (PCR) primers derived from sequences within antibiotic resistance genes used during the course of construction were used to carry out PCR analysis of the strain to demonstrate the absence of these sequences from the final ethanologen strain. The results of these analyses are described in this report.

Materials and Methods

Genomic DNA was extracted from PARENT OF MICROBIAL STRAIN Taurus04.A.54 and MICROBIAL STRAIN Taurus04.A.54 and used as templates for PCR to determine the absence of the Ampicillin-resistance gene. Plasmid pYEplac112 (*Amp* vector control) DNA was used as a positive control for the Ampicillin-resistance gene. The endogenous copy chromosomal gene, *16S*, was used as a positive control. The following PCR primers were used:

Amp

forward primer 5' TCCGTGTCGCCCTTATTCCT 3'

reverse primer 5' TCAGTGAGGCACCTATCTCAGCG 3'

16S

forward primer 5' GCATATCCAATAAGCGGAGGAAAAG 3'

reverse primer 5' GGTCCGTGTTTCAAGACGG 3'

Conditions for PCR were as follows:

Step 1	95 C	2 minutes
Step 2	95 C	15 seconds
Step 3	55 C	30 seconds
Step 4	72 C	45s
Repeat steps 2-4 30 times		
Step 5	72	10 minutes

Reaction products were analyzed on a 0.7% TAE-agarose gel and post-stained using GelRed at dilution of 1:3000.

Results and Conclusions

PCR tests were performed on genomic preparations of PARENT OF MICROBIAL STRAIN Taurus04.A.54 and MICROBIAL STRAIN Taurus04.A.54. The vector pYEplac112, containing the Ampicillin-resistance gene was used to “spike” the genomic preparations of PARENT OF MICROBIAL STRAIN Taurus04.A.54 and MICROBIAL STRAIN Taurus04.A.54 to determine the lower limits of detection. An endogenous single copy chromosomal gene target, *16S*, was used as a positive control for PCR.

The results are shown in Figure 1 and summarized in Table 1. From these results it can be concluded that the ampicillin-resistance gene is absent from the final ethanologen strain MICROBIAL STRAIN Taurus04.A.54.

Sample	Primer	Limit of detection of spiked marker (X)	Genome to marker molar ratio (Y) ¹	Detection sensitivity for single copy gene (Z) ²	Maximum copies per cell ³
Parent of Microbial Strain Taurus04.A.54 plus pYEplac112	<i>Amp</i>	10fg	980	9800fg	0.00098
Microbial Strain Taurus04.A.54 plus pYEplac112	<i>Amp</i>	10fg	980	9800fg	0.00098

Table 1. *Summary of results of PCR analysis for antibiotic resistance markers and calculations of detection sensitivity.*

¹ Based on genome size of 4.9Mb for *Microbial Strain Taurus04.A.54*, 5.0kbp pYEplac112.

² Equals column X times column Y.

³ Equals 10ng of chromosomal DNA used in each PCR reaction divided by column Z.

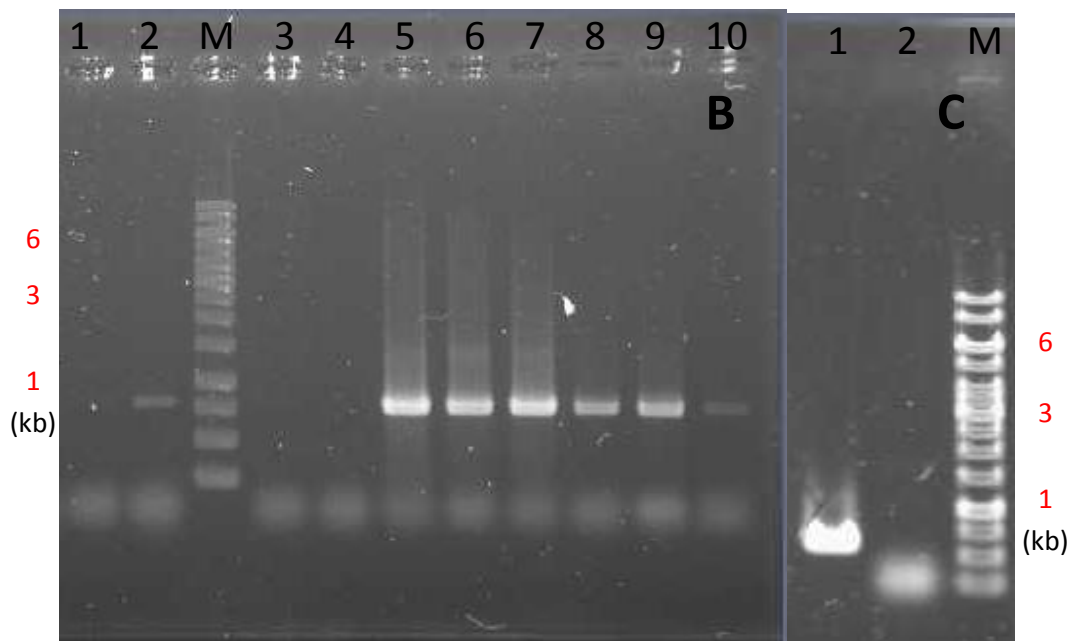
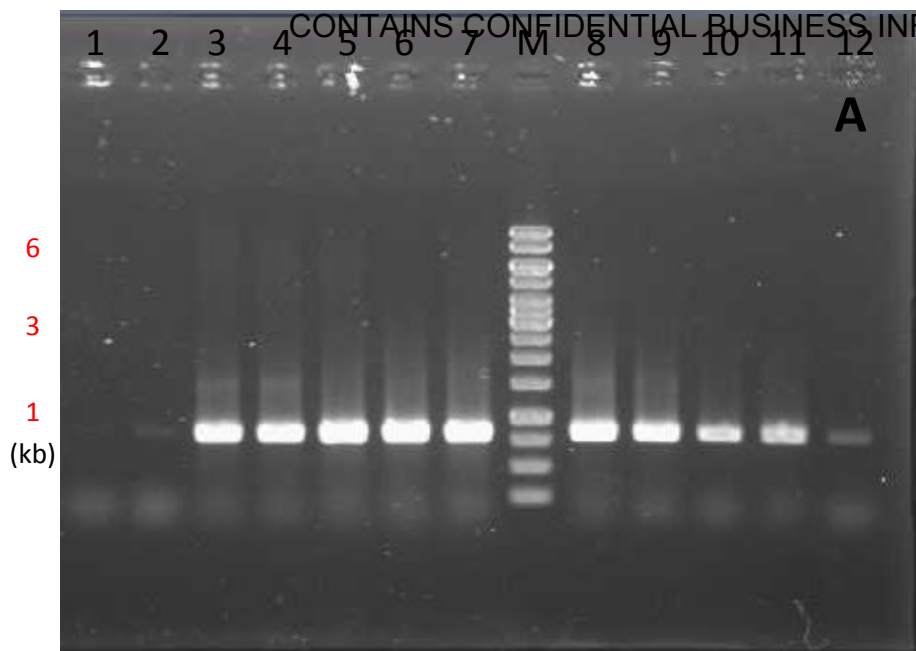


Figure 1. Absence of *Amp* gene in Parent and Microbial Strain Taurus04.A.54. PCR reactions using primers specific to the *Amp* gene and the endogenous chromosomal *16S* gene as positive control. **Upper gel A;** lane 1, Amp deletion strain Taurus04.A.54 (20ng genomic DNA) with *Amp* primers; lane 2, Parent of Amp deletion strain Taurus04.A.54 (20ng genomic DNA) with *Amp* primers; lane 3 Taurus04.A.54 (20ng genomic DNA) plus 10pg YEplac112 vector spike with *Amp* primers; lane 4 Parent of Amp deletion strain Taurus04.A.54 (20ng genomic DNA) plus 10pg YEplac112 vector spike with *Amp* primers; lanes 5, Taurus07.E.48 (20ng genomic DNA) with *Amp* primers; lanes 6, Taurus04.A.48 (20ng genomic DNA) with *Amp* primers; lane 7, Taurus07.E.51 (20ng genomic DNA) with *Amp* primers; lanes 8-12 Amp deletion strain Taurus04.A.54 (100ng genomic DNA) and YEplac112 vector spike (10pg, 1pg, 0.1pg, 10fg, 1fg) with *Amp* primers. **Lower gel B;** lanes 1-2 Amp deletion strain Taurus04.A.54 (20ng, 100ng of Genomic DNA) with *Amp* primers; lanes 3-4, MilliQ water (1µl) with *Amp* primers; lane 5, Taurus04.A.53 (20ng of Genomic DNA); lanes 6-10 Parent of Amp deletion strain Taurus04.A.54 (100ng genomic DNA) with vector spike YEplac112 vector (10pg, 1pg, 0.1pg, 10fg, 1fg) with *Amp* primers. **Lower gel C;** lane 1, Taurus04.A.54 (20ng genomic DNA) with *16S* primers; lane 2, Taurus04.A.54 (10ng genomic DNA) with *Amp* primers; M, molecular weight markers.

